New primers and probes for the detection of HIV

Description

The invention concerns a method for the subtype-independent or/and species-independent detection of HI viruses in a sample and suitable oligonucleotides for this.

The detection of HIV is of major importance in analytical diagnostics. There are a number of detection methods which are based on the immunological detection of HIV-specific antibodies, HIV proteins such as reverse transcriptase or HIV-specific nucleic acids.

Since HIV and its genetic material only occurs in very low concentrations in body fluids, the sensitivity of the detection method is an important factor for the usability of such methods.

For this reason PCR (polymerase chain reaction) which is based on an amplification of the nucleic acids to be detected is now being used to an increasing extent. Applications of this method for the direct detection of HIV are described for example in EP-B-0 200 362 and EP-B-0 201 184.

PCR or the polymerase chain reaction allows the amplification of nucleic acid sections with the aid of oligonucleotides, so-called primers, which hybridize specifically with the nucleic acids to be detected. Amplification products are formed in this process which in turn can be detected with other specific

oligonucleotides, so-called probes.

Prerequisites for a successful PCR for the detection of HIV are on the one hand that the complementary base sequence of the primers used agree as exactly as possible with that of the nucleic acid to be detected so that the hybridization is as specific as possible. However, on the other hand it is also advantageous to be able to amplify as many variants of HIV as possible with the same primers.

Since the discovery of HIV-1 it has been found that there are differences in the nucleic acid sequences of HI viruses of different origins. The various types of HIV-1 are usually referred to as subtypes. At present at least 9 subtypes are known which are referred to as subtypes A to H and O (Human Retroviruses and AIDS, Los Alamos, Natl. Laboratory, Los Alamos, New Mexico, 1994; Publisher G. Meyers et al., I-A-1). No primers or probes have yet been developed that can recognize all known subtypes of HIV-1. In addition it would also be advantageous to be able to also detect HIV-2 and its subtypes with the same primers and probes. At present the HIV-2 subtypes A, B, C and D are known.

In some patent applications oligonucleotide primers and/or probes have been disclosed which, however, are not encompassed by the present application.

WO96/02557 describes general uses of oligonucleotides to inhibit HIV propagation and to detect the virus. However, there is no reference to a suitability for a species-independent or subtype-independent detection.

Primers and probes are described in the European Patent Application EP 0 403 333 which each hybridize with base sequences from conserved regions of the *gag*, *vpr* and *pol* genes of the HIV-1 isolates Bru, Mal and Eli and with corresponding regions of HIV-2 ROD and SIV Mac. In addition primers and probes are disclosed which each hybridize with sections from the *env*, *nef*1, *vif*1 and *vpr* genes of HIV-1 Bru, Mal and Eli and those which hybridize with sections from the *nef*2, *vif*2 and *vpx* genes of HIV-2 ROD and SIV Mac. Although some of these oligonucleotides apparently hybridize independent of species, no statements are made about which subtypes of the individual viruses are recognized.

Further detection methods for HIV are disclosed for example in EP 839 917 which only enables subtypes of HIV-1 to be detected as well as in the applications EP 887 427, WO99/07898 and WO98/58086 which were published later. Primers and probes are disclosed in the European Patent Application EP 727 497 which amplify a sequence section for the *pol* gene of HIV-1 and can thus recognize five subtypes of HIV-1.

EP 0 617 132 also disclose primers and probes for the detection of HIV-1 which are able to distinguish between HIV-1 and its phylogenetically close relatives such as HTLV-II or HIV-2. The oligonucleotides selected in this case hybridize with a number of regions from the HIV genome including LTR and most of the structural genes. However, there are no references to a suitability for a subtype-independent detection.

In addition there are methods in the prior art which use oligonucleotides for a purification by means of

hybridization with target oligos e.g. WO98/27425. Such oligos are, however, not suitable for a subtype-independent detection of HIV.

Although WO90/01069 discloses oligonucleotide pairs for the detection of HIV, these pairs are not used as primers for an enzymatic amplification but rather hybridize with the same strand and themselves represent the sought-after sequence after their linkage.

Since previously there has not yet been a single primer pair that is suitable for a subtype-independent or/and species-independent detection, several primer pairs are now frequently used for the amplification. However, this results, on the one hand, in increased reagent costs and, on the other hand, in a much more complex PCR.

Hence the object of the present invention was to provide a method for the subtype-independent or/and species-independent detection of HI viruses in a sample. In particular it was an object of the invention to provide a method that enables more subtypes of HIV-1 and/or HIV-2 to be detected than was previously possible.

This object is achieved according to the invention by a method for the subtype-independent and/or species-independent detection of nucleic acids of HI viruses in a sample by

hybridizing the nucleic acids with at least one oligonucleotide hybridizing specifically with HIV nucleic acids which contains at least 10 consecutive nucleotides from

(i) a highly conserved region of the LTR region, of the *gag* gene or of the *pol* gene of HIV

represented by one of the sequences shown in SEQ ID NO: 1 to 13,

(ii) a corresponding region of another HI virus isolate,

(iii) a corresponding region of a consensus sequence from several HI virus isolates,

or sequences which are complementary to these.

This object is preferably achieved by a method for the subtype-independent and/or species-independent detection of nucleic acids of HI viruses in a sample by hybridizing the nucleic acids with an oligonucleotide combination comprising two or several oligonucleotides hybridizing specifically with HIV nucleic acids which each contain at least 10 consecutive nucleotides from

(i) a highly conserved region of the LTR region, of the *gag* gene or of the *pol* gene of HIV represented by one of the sequences shown in SEQ ID NO: 1 to 13,

(ii) a corresponding region of another HI virus isolate,

(iii) a corresponding region from a consensus sequence derived from several HI virus isolates,

or sequences that are complementary to these, and performing an amplification step.

The method according to the invention enables more subtypes of HIV-1 and HIV-2 (subtype-independent detection) to be detected than was previously possible with the prior art. Subtype-independent detection means that at least 2 subtypes of a respective species can be detected with a single probe or with a single oligonucleotide combination. As already mentioned above

it has previously not been possible to detect subtypes A, B, C, D, E, F, G, H and O of HIV-1 with a single probe or with a single amplification primer pair composed of two oligonucleotides using conventional detection methods. The method according to the invention now enables at least 7 of 9 subtypes of HIV-1, in particular including subtype O together with other subtypes, to be detected with the aid of very few oligonucleotides and in the best case with the same oligonucleotides. In a preferred embodiment it is possible to detect all previously known 9 subtypes. The subtype-independent test enables the detection of at least 2, preferably at least 3 and most preferably all subtypes A, B, C and D of HIV-2. In addition to the subtype-independent detection it is also possible to detect nucleic acids of HIV-1 and HIV-2 independent of species. Species-independent means that various species of immunodeficient viruses can be detected with the same oligonucleotides e.g. HIV-1 and HIV-2. Preferably at least 7 of the presently known 9 subtypes of HIV-1 and other presently known subtypes of HIV-2 are detected. Particularly preferably it is possible to detect all 9 subtypes of HIV-1 plus further subtypes of HIV-2 and particularly preferably plus all currently known subtypes of HIV-2.

The method according to the invention is based on an amplification of nucleic acid sections of HI viruses with the aid of specific oligonucleotides which can act as primers or as probes.

An oligonucleotide is a single-stranded linear nucleic acid molecule. In general oligonucleotides have 10 to 100 bases. The oligonucleotides according to the invention are preferably 10 to 80, particularly

preferably 10 to 60 and more preferably 10 to 30 and most preferably 20 to 30 nucleotides long. As with nucleic acids one distinguishes here between oligodeoxyribonucleotides and oligoribonucleotides; however, oligoribonucleotides also include compounds in which the hydrogen of the hydroxy group is replaced by organic residues such as an allyl group. Such compounds have been known for a long time to a person skilled in the art. In addition the term "oligonucleotide" can also refer to molecules in which the sugar phosphate backbone is replaced by a peptide backbone. This group of compounds is referred to as PNA. A common feature of all oligonucleotides is that they have bases on the backbone which are able to form hydrogen bonds with complementary bases. The bases include the natural bases A, G, C, T and U and also artificial bases such as deaza-G.

An oligonucleotide primer is an oligonucleotide which can hybridize with a second likewise single-stranded nucleic acid and subsequently be extended with nucleoside triphosphates by a suitable enzyme e.g. a DNA polymerase along the second nucleic acid which serves as a template such that a double-stranded nucleic acid is formed. Hence the primers normally have a hydroxyl group on the 3' C atom of the sugar at the 3' end i.e. at the end where the nucleotide building blocks are attached.

An oligonucleotide combination comprises several oligonucleotides and it preferably comprises a primer pair or a combination of primers and probes such as a primer pair and a probe. A primer pair is composed of two oligonucleotide primers which allow the, preferably enzymatic, amplification of a certain section of a nucleic acid. Preferably both primers of the primer pair hybridize with different strands of the original nucleic

acid such that the respective extension products overlap each other. As a result each primer can hybridize with the extension product of the other primer and a section between the two primers is multiplied as an amplification product. So-called probes are also single-stranded oligonucleotides which, although also acting as primers, are mainly used to specifically hybridize with already amplified nucleic acid sections in order to thus enable a nucleic acid detection. The name oligonucleotide thus encompasses the term primer and probe which only differ in their function. The oligonucleotides used in the method according to the invention can also contain marker groups such as radioactive labels or fluorescent labels. It is especially the probes which have labels.

Amplification is understood as a multiplication of nucleic acids or sections of nucleic acids. A known amplification method is the already mentioned PCR. In this method a normally double-stranded DNA molecule is firstly denatured i.e. is split into its single strands. Then the amplification primers are added under conditions which allow hybridization of the primers with the target DNA sequence. The primers are then extended along the nucleic acid template with the aid of a polymerase enzyme and nucleotide building blocks. Subsequently the newly formed double-stranded nucleic acids are again denatured and a new polymerase cycle begins. Conventional PCR methods typically use between 25 and 40 cycles. An amplification in the sense of the invention can also include necessary preparation steps for the nucleic acid amplification such as the denaturation of the double-stranded DNA.

In this application the term "hybridization" denotes the

joining of two complementary nucleic acid single strands to form a double strand. The single strands do not have to be 100 % complementary for this but can have deviations in their base sequence. In order to achieve a suitable hybridization for the method according to the invention the single strands, in this case the oligonucleotides, must, however, be sufficiently specific under the prevailing hybridization conditions.

In order to ensure the advantages mentioned above of the method according to the invention described here, the oligonucleotides that are used must fulfil at least two conditions. On the one hand they must be sufficiently specific to exclusively hybridize with nucleic acids which are derived from HI viruses. On the other hand these viruses vary very greatly in some genome regions i.e. there are relatively large differences in the base sequence. On the basis of these differences one distinguishes on the one hand between HIV-1 and HIV-2 as well as between so-called subtypes which represent relatively closely related strains of the same virus. With reference to the oligonucleotides of the present invention this means that these must also be able to not only recognize a particular virus or a particular subtype but they must also have a base sequence that allows hybridization with as many subtypes of HIV-1 or HIV-2 as possible or even with all known subtypes or even hybridization with both.

For this purpose one usually selects oligonucleotides from relatively conserved regions of the genome of the viruses to be detected and then uses the respective complementary sequence. Some highly conserved regions are already known from the prior art (see above). Conserved regions are sections of nucleic acid on the

genome of HIV which, compared to the remainder of the genome, only have very slight differences in the base sequence. This is also referred to as base identity which is expressed in percent or as homology.

Surprisingly oligonucleotides were found in the present invention which allow a subtype-independent detection as well as a species-independent detection of HIV. These oligonucleotides hybridize with newly discovered highly conserved regions of HIV. These are located in the LTR region, the *gag* gene and the *pol* gene. These regions are relatively small but, having an average length of 50 to 100 nucleotides, they allow hybridization with one or several oligonucleotides. As in most of the cited documents of the prior art the location of the regions of the HIV genome in this application is based on the numbering of the HIV-1 isolate HXB2 as published in : Wong-Staal et al., Nature 313, 277-284 (1985). The sequences of these new highly conserved regions are in each case shown in SEQ ID NO. 1 to 13, the sequences of which refer to the sequence of HIV-1 HXB2, accession number K03455 of the HIV sequence data base (http://HIV-web.lanl.gov./). The position of the highly conserved sequences is as follows:

SEQ ID NO.1: LTR region, position 504-565, length 62 nucleotides,

SEQ ID NO.2: *gag* gene, position 761-822, length 62 nucleotides,

SEQ ID NO.3: *gag* gene, position 1786-1847, length 62 nucleotides,

SEQ ID NO.4: *pol* gene, position 2307-2360, length 54 nucleotides,

SEQ ID NO.5: *pol* gene, position 2376-2434, length 59 nucleotides,

SEQ ID NO.6: *pol* gene, position 2568-2632, length 65 nucleotides,

SEQ ID NO.7: *pol* gene, position 3093-3145, length 53 nucleotides,

SEQ ID NO.8: *pol* gene, position 4131-4207, length 77 nucleotides,

SEQ ID NO.9: *pol* gene, position 4333-4399, length 67 nucleotides,

SEQ ID NO.10: *pol* gene, position 4638-4696, length 59 nucleotides,

SEQ ID NO.11: *pol* gene, position 4884-4984, length 101 nucleotides,

SEQ ID NO.12: *pol* gene, position 5034-5095, length 62 nucleotides,

SEQ ID NO.13: *pol* gene, position 4410-4506, length 97 nucleotides,

The oligonucleotides that are suitable according to the invention preferably have base sequences that are

located within the above-mentioned highly conserved regions or their complementary sequences.

The term "overlapping" should be understood to mean that each of the oligonucleotides according to the invention overlaps at least 10 consecutive bases from one of the highly conserved regions.

The oligonucleotides preferably each overlap one of the regions of SEQ ID NO: 4, 5, 9, 10 and 13, preferably 4, 5, 9 and 10. In another preferred embodiment the oligonucleotides each overlap one of the regions of SEQ ID NO: 6, 8, 10, 11 and 13.

Although the highly conserved regions are stated with reference to a single HIV-1 virus isolate, the term "highly conserved region" of course applies to more than a single specific sequence and thus includes all corresponding regions from various HIV strains or isolates that are related to HIV-1 or HIV-2. Suitable oligonucleotide sequences according to the invention can also have a base sequence which represents a consensus sequence from highly conserved regions of several HI virus isolates or strains. This means that for example a base sequence of several bases corresponds to one HI virus isolate and another base sequence in the same oligonucleotide corresponds to another HI virus isolate. The oligonucleotide then contains heterologous base sequences. The oligonucleotides preferably each contain at least 10 consecutive nucleotides from one of the above-mentioned regions. More preferably they contain 15 to 30 such nucleotides.

The method according to the invention comprises at least

the following steps:

(a) contacting a sample with the oligonucleotide(s) under such conditions that the oligonucleotide(s) hybridizes/hybridize with the HIV nucleic acids selected from HIV-1 or/and HIV-2 that are present in the sample,

(b) determining the presence or/and the amount of HIV nucleic acids in the sample.

An amplification step is preferably carried out in step (b).

In a preferred embodiment of the inventive method the subtype-independent or/and species-independent detection of HI virus nucleic acids is made possible by using at least two oligonucleotides according to the invention. These oligonucleotides are preferably used as amplification primers in such a way that they for example each hybridize near to one end of the highly conserved regions and thus generate an amplification product in the amplification (preferably PCR) which corresponds to a section of the respective highly conserved region. This amplification product and thus the HIV-specific nucleic acid can then be detected with the aid of a probe which is either one of the oligonucleotides used as a primer or an additional oligonucleotide which hybridizes within the amplified sequence. The advantage of this preferred embodiment is that a single oligonucleotide or primer pair is sufficient to detect HI viruses independent of subtype or/and species.

Instead of using a probe to detect the amplification product, DNA-binding reagents such as a DNA-binding dye

(e.g. Sybergreen) can also for example be used for the detection (WO97/46707).

According to another preferred embodiment of the method of the invention oligonucleotide combinations or primer pairs are used of which only one oligonucleotide primer is located within one of the conserved regions whereas the other primer is located outside such that the amplification product that is generated in this manner is only partially composed of a base sequence from one of the highly conserved regions. The latter primer is preferably subtype-specific and/or species-specific and thus such combinations can be used to specifically detect subtypes or the species.

In this case preferably two or more primer pairs are used as the oligonucleotide combination of which at least two oligonucleotides each contain at least 10 consecutive nucleotides from a sequence (i), (ii), (iii) or sequences that are complementary thereto as described above. The totality of the primer combinations thus allows a detection of HI viruses that is independent of subtype or/and species.

In a further preferred embodiment a suitable oligonucleotide combination for the method according to the invention comprises two, preferably three oligonucleotides (e.g. a primer pair or a primer pair plus probe) which each contain at least 10 nucleotides from

(i) the same highly conserved region of the *gag* gene or of the *pol* gene of HIV represented by one of the sequences shown in SEQ ID NO:1 to 13,

(ii) a corresponding region from another HI virus

isolate,

(iii) a corresponding region from a consensus sequence derived from several HI virus isolates,

or sequences that are complementary thereto.

The at least one and preferably the at least two oligonucleotide(s) are preferably selected such that they enable a subtype-independent detection of HIV-1 i.e. nucleic acids of at least 7 of the subtypes A, B, C, D, E, F, G, H and O of HIV-1 and preferably all subtypes are detected.

For the subtype-independent detection it is preferable to use at least one and preferably at least two oligonucleotide(s) which each contain at least 10 consecutive nucleotides from

(i) a highly conserved region of the LTR, *gag* gene or *pol* gene of HIV represented by one of the sequences shown in SEQ ID NO: 1, 2, 3, 4, 5, 6, 8, 9, 10, 12 and 13,

(ii) a corresponding region of another HI virus isolate,

(iii) a corresponding region from a consensus sequence derived from several HI virus isolates,

or sequences that are complementary thereto.

For the subtype-independent or/and species-independent detection of HIV-1 and HIV-2 it is preferable to use at least one and preferably at least two oligonucleotide(s) which each contain at least 10 consecutive nucleotides from

(i) a highly conserved region of the LTR, *gag* gene or *pol* gene of HIV represented by one of the sequences shown in SEQ ID NO: 1, 2, 3, 4, 5, 7, 9, 10 and 13,

(ii) a corresponding region of another HI virus isolate,

(iii) a corresponding region from a consensus sequence derived from several HI virus isolates,

or sequences that are complementary thereto.

It surprisingly turned out that very special oligonucleotides are particularly suitable for the method according to the invention. These oligonucleotides are represented by the sequences shown in SEQ ID NO: 14 to 25. As mentioned above these oligonucleotides can be composed of natural or synthetic nucleic acid building blocks or even of the already mentioned PNA. Preferably at least one of the oligonucleotides used in the method according to the invention carries one or several labels. Suitable labels are fluorescent labels or radioactive labels such as [^{32}P]-labelled nucleotides.

A further subject matter of the invention is an oligonucleotides which contains at least 10 consecutive nucleotides from

(i) a highly conserved region of the *pol* gene of HIV represented by one of the sequences shown in SEQ ID NO: 4, 5, 9 or 10,

(ii) a corresponding region of another HI virus isolate,

(iii) a corresponding region from a consensus sequence derived from several HI virus isolates, or sequences that are complementary thereto or contain one of the sequences shown in SEQ ID NO: 14 to 25.

The oligonucleotide according to the invention preferably comprises one of the oligonucleotides shown in SEQ ID NO. 14 to 25. The length of the

oligonucleotide according to the invention is preferably 10 to 80 nucleotides. It particularly preferably has about 20 to 30 bases and has a GC content between 40 and 60 %. In addition it is advantageous when the oligonucleotides have no self-complementarity at the 3' end and in addition have no CG run at the 3' end. A GC run is a base sequence which is mainly or exclusively composed of the bases C and G. In addition the oligonucleotides should contain no palindromes. The oligonucleotides according to the invention have preferably no more than 2 mismatches with the corresponding sequences which hybridize with them at the same positions in all subtypes. There are preferably no mismatches at the 3' end of an oligonucleotide according to the invention with nucleotidic acids of the subtypes A, B, C, D, E, F, G, H and O of HIV-1 and/or of subtypes A, B, C and C of HIV-2.

In the case of primer pairs the primers are each selected such that the 5' ends of the primers are positioned no more than 80 bases from one another with respect to the regions in which the primers hybridize on the nucleic acid to be detected. Particularly preferred primer pairs are those in which there is an additional HIV-specific sequence within the region amplified by the primers. This sequence can then be preferably used to detect the amplification products with the aid of a probe which is specific for it. The oligonucleotides according to the invention are preferably provided with at least one of the above-mentioned marker groups.

Thus a further aspect of the present invention is also a combination of two or more oligonucleotides which both have the above-mentioned inventive properties and which in their entirety are suitable for the subtype-

independent and/or species-independent detection of HI viruses.

The invention additionally encompasses combinations of oligonucleotides. Combinations of at least two oligonucleotides are preferred which each contain at least 10 consecutive nucleotides from

(i) the same highly conserved region of the LTR region, the *gag* gene or *pol* gene of HIV represented by one of the sequences shown in SEQ ID NO: 1 to 13,

(ii) a corresponding region of another HI virus isolate,

(iii) a corresponding region from a consensus sequence derived from several HI virus isolates,

or sequences that are complementary thereto.

Yet a further aspect of the present invention is a combination of several oligonucleotides where at least two oligonucleotides according to the invention are present and further oligonucleotides which each contain a specific sequence for a single subtype of HIV-1 or/and HIV-2 wherein the entirety of the oligonucleotides in the oligonucleotide combination allows a subtype-independent or/and species-independent detection of HI viruses.

A further object of the present invention is the provision of a reagent kit which contains at least one oligonucleotide according to the invention or an oligonucleotide combination according to the invention as primers or as probes for the detection of HI viruses or their nucleic acids as well as suitable means for carrying out a hybridization and amplification of nucleic acids in a sample.

The invention additionally concerns the use of oligonucleotides or oligonucleotide combinations as primers or/and probes for the detection of HI viruses and especially for the subtype-independent and/or species-independent detection.

It is intended to elucidate the present invention in an illustrative but in no way exhaustive manner by the following examples.

Examples

Example 1

Sensitivity, specificity and dynamic measuring range based on a serial dilution of HIV plasma

In order to examine blood samples, RNA was isolated from HIV-positive plasma with an initial concentration of 15,000 genome equivalents (geq) HIV per ml. This plasma was diluted successively by a factor of 10 in negative plasma and, after sample preparation, each was amplified in duplicate determinations using the corresponding primer pairs. A HIV-negative plasma and water served as controls. In order to determine the specificity a HBV-positive and a HCV-positive plasma was also processed. All samples were measured after amplification (ECL detection, Elecsys®1010).

1. Sample preparation:

Firstly 420 µl plasma was mixed with 80 µl proteinase K (25 mg/ml) and vortexed for several seconds. Then 500 µl lysis buffer (5.4 M guanidinium thiocyanate, 10 mM urea, 10 mM Tris-HCl, 20 % Triton X100, pH 4.4) which contained 1 µg carrier RNA (polyA/ml) was added. The mixture was vortexed and subsequently shaken for 10 minutes at room

temperature. Then 500 µl isopropanol-MGP was added (6 mg magnetic glass particles in isopropanol). The mixture was again vortexed and subsequently shaken for 20 minutes at room temperature. The MGPs were separated magnetically from the solution. The supernatant was removed and discarded. 750 µl wash buffer (20 mM NaCl, 20 mM Tris-HCl, pH 7.5, 70 % ethanol) was added, the MGPs were resuspended by vortexing and a magnetic separation was again carried out. The wash process was repeated a total of 5 times and finally 100 µl DEMC water was added for elution. It was shaken for 15 minutes at 80°C and then an addition magnetic separation was carried out. 10 µl of the eluate was used for an RT-PCR.

2. Primers and probes which were used:
Primers and probes that were used are shown in the following table 1 which also shows their respective highly conserved region, position in the genome and the amplification product produced with the primer pairs.

Table 1

Primer	highly conserved region	position	amplicon
SK 462* SK 431* SK 102		1359-1388 (30) 1474-1500 (27) 1402-1421 (20)	142 bp
RAR 1032* RAR 1033* RAR 1034*		2961-2992 (32) 3097-3129 (33) 2997-3031 (35)	169 bp
GH A2F** (SEQ ID NO. 14) GH A2R** (SEQ ID NO. 15) GH A2P** (SEQ ID NO. 16)	SEQ ID NO. 8	4143-4162 (20) 4180-4205 (26) 4162-4179 (18)	63 bp
GH A3F** (SEQ ID NO. 17) GH A3R (SEQ ID NO: 18) GH A3P (SEQ ID NO. 19)	SEQ ID NO. 10	4644-4663 (20) 4677-4702 (26) 4663-4677 (15)	59 bp
GH A4F** (SEQ ID NO. 20) GH A4R** (SEQ ID NO. 21) GH A4P** (SEQ ID NO. 22)	SEQ ID NO. 11	4889-4912 (24) 4932-4951 (20) 4913-4931 (19)	63 bp
GH A6F** (SEQ ID NO. 23) GH A6R** (SEQ ID NO. 24) GH A6P (SEQ ID NO. 25)	SEQ ID NO. 13	4412-4437 (26) 4461-4485 (25) 4438-4460 (23)	74 bp

* These primers are all published primers and probes from Roche

** These primers are new primers from the *pol* region of the HIV genome and correspond to the highly conserved regions shown in table 1.

3. Amplification mix and thermocycler protocol for the RT-PCR:
Master mix:

Reagents	**Final concentration in the master mix**
5 x bicin buffer	1 x
MnOAc	2.5 mM
dNTPs (incl. dUTP)	200 µM/600 µM
forwards primer	0.3 µM
reverse primer (biotinylated)	0.3 µM
Tth polymerase	10 units
UNG	2 units
total volume:	100 µl

Cycler:

	10 minutes, 37°C: UNG decontamination
	30 minutes, 60°C: reverse transcription
	30 seconds, 95°C: denaturation
5 cycles	15 seconds, 95°C: denaturation
	20 seconds, 50°C: hybridization/elongation
30 cycles	15 seconds, 94°C: denaturation
	20 seconds, 60°C: hybridization/elongation
	7 minutes, 72°C: final elongation
	keep at 50°C

4. Detection:

The entire detection reaction was fully automated with the aid of an Elecsys®1010 automated analyser.

Firstly 10 µl amplificate and 35 µl denaturation solution (BM-ID-No. 1469053, Boehringer Mannheim) were removed. They were incubated for 5 minutes at 37°C in a reaction vessel and then 120 µl hybridization solution (BM-ID-No. 1469045, Boehringer Mannheim to which 25 ng/ml ruthenium-labelled probe was added) was added. It was again incubated for 30 minutes at 37°C. Then 35 µl of an Elecsys® SA magnetic bead solution was added (BM-ID-No. 1719556, Boehringer Mannheim). The solution was incubated for 10 minutes at 37°C. The electrochemiluminescence of 120 µl of the reaction mixture was measured in the Elecsys® 1010 measuring cell. The appropriate ruthenium-labelled probes were used according to table 1 for hybridization.

5. Results

Result (ECL counts x 100):

Template	SK primer	RAR primer	GH-A2	GH-A3	GH-A4	GH-A6
HIV 15000 copies/ml	5763	294	5786	4209	7981	6809
HIV 1500 copies/ml	626	38	724	466	899	999
HIV 150 copies/ml	184	14	86	164	117	122
HIV 15 copies/ml	58	9	13	27	25	10
HIV 1.5 copies/ml	49	9	14	32	14	10
HIV-negative plasma	70	9	22	38	16	11
HCV-positive plasma	49	9	5	58	16	10
HBV-positive plasma	37	9	5	81	17	10
water	12	9	16	35	15	10

The signals of the new primers have a similar sensitivity to the references. There is a very good signal gradation within the serial dilution.

The increased background with the primer pairs SK and GH-A3 is presumably due to a non-optimal amplification protocol.